Freezing and chemical preservatives alter the stable isotope values of carbon and nitrogen of the Asiatic clam (*Corbicula fluminea*)

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Abstract We tested the impacts of most common sample preservation methods used for aquatic sample materials on the stable isotope ratios of carbon and nitrogen in clams, a typical baseline indicator organism for many aquatic food web studies utilising stable isotope analysis (SIA). In addition to common chemical preservatives ethanol and formalin, we also assessed the potential impacts of freezing on $\delta^{13}C$ and $\delta^{15}N$ values and compared the preserved samples against freshly dried and analysed samples. All preservation methods, including freezing, had significant impacts on $\delta^{13}C$ and $\delta^{15}N$ values and the effects in general were greater on the carbon isotope values (1.3–2.2‰ difference) than on the nitrogen isotope values (0.9–1.0‰ difference). However, the impacts produced by the preservation were rather consistent within each method during the whole 1 year experiment allowing these to be accounted for, if clams are intended for use in retrospective stable isotope studies.

Keywords Formalin · Freezing · Ethanol · Preservation · Stable isotope analysis

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Stable isotope analysis (SIA) from preserved and archived sample materials offers unique possibilities for reconstructing historical food webs and for retrospective ecosystem studies. Many universities, museums and research institutions hold collections of preserved sample materials which potentially could be turned into valuable long-term ecosystem data sets. Analyses of carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) stable isotope ratios in sample materials (expressed relative to a standard as $\delta^{13}C$ and $\delta^{15}N$ values) provide information about food web structure, consumer trophic positions as well as energy sources and pathways within ecosystems (Peterson & Fry, 1987; Fry, 2006). However, to date surprisingly few studies have effectively utilised such source of information, perhaps reflecting some prevailing uncertainties about preservation impacts on stable carbon and nitrogen isotope values in sample materials.

In general, however, many reported impacts of preservatives have been relatively small, particularly those on δ^{15}N values (~1‰), suggesting that preserved samples can potentially be utilised in historical food web studies, provided that any impacts can reliably be accounted for. But many contradictions and variability in results and interpretations still exist (see Barrow et al., 2008; Ventura & Jeppesen, 2009 for recent literature summaries on preservation impacts). For example, Feuchtmayr & Grey (2003) reported elevated δ^{13}C values in zooplankton after preservation in formalin, whereas many other studies on aquatic animals such as fish, zooplankton and macroinvertebrates (e.g. Mullin et al., 1984; Bosley & Wainright, 1999; Kaehler & Pakhomov, 2001; Edwards et al., 2002; Sarakinos et al., 2002; Syväranta et al., 2008b) have reported opposite impacts. Similarly, some studies on aquatic animals reported strong and significant impacts on δ^{15}N values (e.g. Sarakinos et al., 2002; Feuchtmayr & Grey, 2003; Kelly et al., 2006), while some only little or no impacts at all (e.g. Mullin et al., 1984; Ogawa et al., 2001; Syväranta et al., 2008b). The magnitude of these reported impacts of preservatives on stable carbon and nitrogen values has been highly variable and range, for both δ^{13}C and δ^{15}N values, from no impact to over 2‰ difference between control and preserved samples. Similarly contradicting results are reported after ethanol preservation of fish, zooplankton and macroinvertebrate samples (e.g. Kaehler & Pakhomov, 2001; Sarakinos et al., 2002; Feuchtmayr & Grey, 2003; Syväranta et al., 2008b) but fewer studies have considered testing for fixation in formalin and subsequent transfer to ethanol preservation, a technique which is often employed in institutions and museums (Bosley & Wainright, 1999; Carabel et al., 2009), particularly for invertebrate samples. Freezing is likely the most common method to preserve samples for SIA when immediate drying is not possible, but not all studies have considered the potential impacts of freezing of sample on stable isotope ratios. Among the studies that have tested the effects of freezing on aquatic animal tissues and/or whole organisms, some found no impacts (Bosley & Wainright, 1999; Kaehler & Pakhomov, 2001; Sweeting et al., 2004) while others found significant and even strong impacts (Feuchtmayr & Grey, 2003; Dannheim et al., 2007; Barrow et al., 2008, this study) on stable carbon and nitrogen isotope values. In addition, impacts of preservatives seem to be highly taxa-specific and preservation studies are often ran as pilot experiments for other studies and may therefore suffer from extremely low number of replicates or incomplete replication, taxonomic variation within replicates and/or using frozen samples as control.

Here we report results from experimental testing of the impacts of chemical preservatives (ethanol and formalin) and freezing on the stable carbon and nitrogen isotope ratios of a freshwater clam [the Asiatic clam *Corbicula fluminea* (O. F. Müller, 1774)]. Clams are long-lived primary consumers that are often preferred as baseline indicators in SIA studies of aquatic ecosystems (Post, 2002) and are isotopically shown to closely match the seasonal averages of zooplankton primary consumers (Syväranta et al., 2008a). *C. fluminea* is a highly invasive species and can form dense clam beds both in European and North American river and lake bottoms. Albeit invasive, it may serve as a valuable baseline indicator for many present and future SIA studies. Historical samples of clams are likely to be available from many institutional collections which potentially can be used to set "historical stable isotope baselines" for comparisons between and within ecosystems in long-term SIA studies. Despite their apparent importance for stable isotope ecology, only two past studies were identified to specifically test for preservation impacts on clams (Sarakinos et al., 2002; Carabel et al., 2009). Our aim was to provide precise evaluation of the effects of the typical chemical preservatives and freezing methods on δ^{13}C and δ^{15}N values of *C. fluminea* on a long-term basis by preserving samples up to 1 year and comparing the isotope values to freshly dried and analysed samples.

Clam samples were collected with hand nets from the Roques-sur-Garonne area of the river Garonne in Toulouse, southwestern France, in December 2008 and immediately taken to the laboratory for cleaning and processing. Samples were divided into those dissected, dried and analysed immediately (control) and those preserved for 1 week, 1, 6, or 12 months either by freezing (at −20°C) or in ethanol (70%), formalin (4%) or by first fixing in formalin for 2 days and then transferring to ethanol (formalin–ethanol), each group having 5–6 replicate samples. Clams were preserved attached to their shells in all treatments, submerged in the preservative in plastic vials during ethanol/formalin preservation at room temperature

and in plastic vials without excess water when frozen. After all preservation treatments, samples were carefully rinsed several times in clean tap water and the foot tissue dissected and cleaned. Only the foot tissue was used for SIA and all samples were oven dried (at 60°C for 48 h) and ground into a fine homogeneous powder using a mixer mill (Retsch MM 200).

Approximately 0.2 mg of sample material was accurately weighed into tin cups and stable isotope ratios of carbon and nitrogen were analysed after combustion in a Carlo Erba NC2500 elemental analyser (Carlo Erba, Milan, Italy) with a Finnigan Mat Delta XP isotope ratio mass spectrometer (Thermo Finnigan, Bremen, Germany). Each analysed sample corresponds to a single individual clam. Stable isotope ratios are expressed as parts per thousand (‰) delta values (δ^{13}C or δ^{15}N‰) referred to the international standards for carbon (PeeDee Belemnite) and nitrogen (atmospheric nitrogen). Data were inspected and corrected using working standards (bass muscle, bovine liver, nicotinamide; SD <0.2‰ for both δ^{13}C and δ^{15}N) that were previously calibrated against International Atomic Energy Agency (IAEA) standards. All stable isotope analyses were performed at the Stable Isotopes in Nature Laboratory, University of New Brunswick, Canada.

Impacts of preservation method and preservation time on stable isotope values were analysed by comparing preserved samples to control samples using analysis of variance (ANOVA) with Tukey's pairwise comparisons tests after testing for data normality and variance homogeneity. All statistical analyses were done using a SPSS 13.0 for Windows software package (SPSS Inc., 2004).

Preservation increased mean (±SD) δ^{13}C and δ^{15}N values by 1.8 ± 0.5‰ ($F_{4,108} = 163.9$, $P < 0.001$) and 1.0 ± 0.3‰ ($F_{4,108} = 46.4$, $P < 0.001$), respectively, as compared to the control samples, which were dried immediately after collection. Duration of the preservation did not significantly affect the difference in stable isotope values or C:N ratios between control and preserved samples, except for δ^{13}C values in samples preserved with formalin, which after 6 and 12 months of preservation had significantly lower δ^{13}C values compared to 1 week preservation ($F_{3,23} = 12.3$, $P < 0.001$; Fig. 1). In all other preserved samples the impacts were evident already after 1 week of preservation.

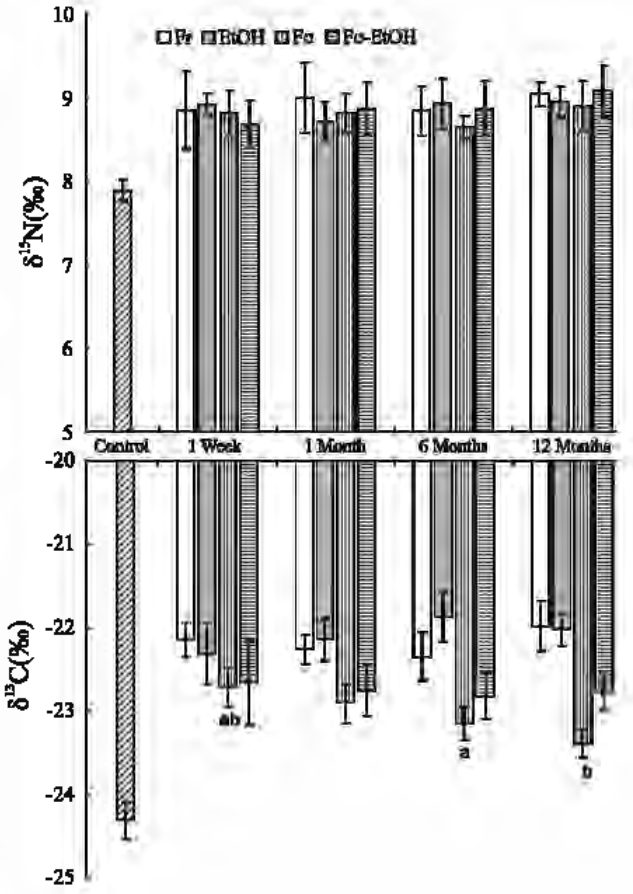

Fig. 1 Changes in δ^{15}N (*upper panel*) and δ^{13}C (*lower panel*) values after a week, a month, 6 and 12 months preservation time by freezing (Fr) or in ethanol (EtOH), formalin (Fo) or formalin–ethanol (Fo–EtOH) against the control samples. Stable isotope values significantly differed from control values after all treatments. *Bars* represent means (±SD) of 5–6 replicate values and *letters* indicate significant differences ($P < 0.05$) after different preservation times in formalin

Averaging for the entire preservation period (Fig. 2; Table 1), freezing and ethanol preservation had significantly ($F_{3,92} = 49.8$, $P < 0.001$) more impact on δ^{13}C values elevating the mean (±SD) by 2.1 ± 0.3 and 2.2 ± 0.3‰, whereas formalin and formalin–ethanol elevated the values by 1.3 ± 0.3 and 1.6 ± 0.3‰, respectively. The impact of preservation on δ^{15}N values was similar for all preservatives as freezing, ethanol, formalin and formalin–ethanol elevated the δ^{13}C values by 1.0 ± 0.3, 1.0 ± 0.2, 0.9 ± 0.2 and 1.0 ± 0.3‰, respectively. Only ethanol preservation significantly affected the C:N ratios in sample materials by lowering the ratios from 3.9 ± 0.4 in control samples to 3.5 ± 0.4. However, the elemental compositions of preserved samples changed during all treatments (Table 1). Carbon content (C%)

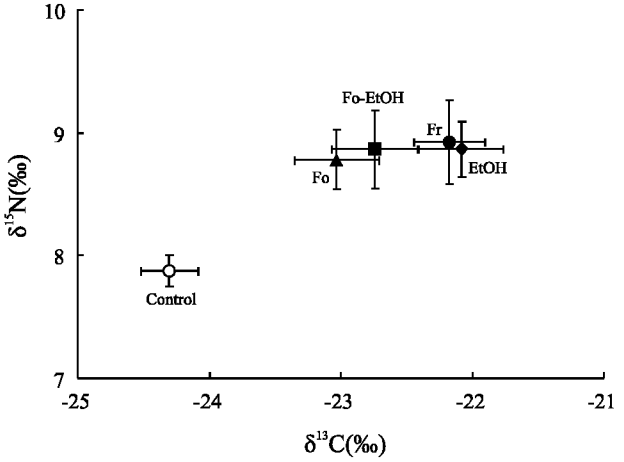

Fig. 2 Stable isotope biplot of mean (±SD) differences in $\delta^{13}C$ and $\delta^{15}N$ values between control and preserved samples (*Fr* freezing, *EtOH* ethanol, *Fo* formalin, *Fo–EtOH* formalin–ethanol) for the whole 12-months study period

became significantly lower (by 1.7–2.2 units) as compared to control samples after the treatments ($F_{4,108} = 6.1$, $P < 0.001$), but there were no differences among the treatments or treatment duration. Similarly the N% became lower after treatments by 0.9–0.4 units ($F_{4,108} = 36.4$, $P < 0.001$), except in ethanol preservation, which significantly increased the N% by 1.0 unit ($P = 0.001$).

Our results on the impacts of preservatives on stable isotope ratios showed clear and significant impacts on the $\delta^{13}C$ and $\delta^{15}N$ values of clams, a typical organism used in various ecological SIA studies to set isotope baseline values. Also freezing, perhaps the most common preservation method, significantly elevated both $\delta^{13}C$ and $\delta^{15}N$ values, in contrast to the findings of Bosley & Wainright (1999), Kaehler & Pakhomov (2001) and Sweeting et al. (2004). However, contrary to the impacts of freezing on zooplankton (Feuchtmayr & Grey, 2003) and macrozoobenthos (Dannheim et al., 2007) samples, our clam samples became significantly ^{13}C-enriched (i.e. higher $\delta^{13}C$ values) after freezing at −20°C. Impact of freezing on $\delta^{15}N$ values was similar to previous findings with around 1‰ increase (Feuchtmayr & Grey, 2003; Dannheim et al., 2007). These differences could partly be explained by the freezing methods employed (e.g. −80 vs. −20°C) and by taxa-specific differences. Clams are protected by their shell but when removed from these they are soft-bodied and easily loose body fluids compared to organisms protected by chitinous exoskeletons.

Table 1 Mean difference (±SD) of preserved sample $\delta^{13}C$, $\delta^{15}N$, C:N, C% and N% values compared to control samples after each tested preservation period

	Freezing	Ethanol	Formalin	Fo + EtOH
$\delta^{13}C$				
1 week	+2.2 ± 0.2	+2.0 ± 0.4	+1.6 ± 0.2	+1.6 ± 0.5
1 month	+2.0 ± 0.2	+2.2 ± 0.3	+1.4 ± 0.2	+1.6 ± 0.3
6 months	+2.0 ± 0.3	+2.4 ± 0.3	+1.2 ± 0.2	+1.5 ± 0.3
12 months	+2.3 ± 0.3	+2.3 ± 0.2	+0.9 ± 0.2	+1.5 ± 0.2
Mean	+2.1 ± 0.3	+2.2 ± 0.3	+1.3 ± 0.3	+1.6 ± 0.3
$\delta^{15}N$				
1 week	+1.0 ± 0.5	+1.0 ± 0.1	+0.9 ± 0.3	+0.8 ± 0.3
1 month	+1.1 ± 0.4	+0.8 ± 0.2	+0.9 ± 0.2	+1.0 ± 0.3
6 months	+1.0 ± 0.3	+1.0 ± 0.3	+0.8 ± 0.1	+1.0 ± 0.3
12 months	+1.2 ± 0.1	+1.1 ± 0.2	+1.0 ± 0.3	+1.2 ± 0.3
Mean	+1.0 ± 0.3	+1.0 ± 0.2	+0.9 ± 0.2	+1.0 ± 0.3
C:N				
1 week	+0.1 ± 0.1	−0.3 ± 0.1	+0.1 ± 0.1	+0.1 ± 0.2
1 month	0.0 ± 0.2	−0.3 ± 0.3	+0.1 ± 0.1	0.0 ± 0.2
6 months	+0.2 ± 0.1	−0.6 ± 0.0	+0.1 ± 0.1	−0.2 ± 0.1
12 months	+0.2 ± 0.1	−0.5 ± 0.0	+0.2 ± 0.1	−0.1 ± 0.2
Mean	+0.1 ± 0.2	−0.4 ± 0.2	+0.1 ± 0.1	0.0 ± 0.2
C%				
1 week	−3.1 ± 0.5	−0.4 ± 1.2	−1.6 ± 0.7	−1.5 ± 1.8
1 month	−2.0 ± 1.8	−2.1 ± 1.4	−1.5 ± 1.0	−2.0 ± 0.9
6 months	−2.1 ± 1.2	−2.1 ± 1.1	−1.5 ± 0.5	−2.1 ± 0.2
12 months	−1.5 ± 1.1	−2.6 ± 0.9	−2.1 ± 1.5	−2.8 ± 4.1
Mean	−2.2 ± 1.3	−1.7 ± 1.4	−1.7 ± 1.0	−2.1 ± 2.3
N%				
1 week	−1.0 ± 0.5	+0.8 ± 0.3	−0.9 ± 0.5	−0.8 ± 0.9
1 month	−0.4 ± 0.7	+0.7 ± 1.1	−0.8 ± 0.4	−0.6 ± 0.6
6 months	−1.1 ± 0.4	+1.7 ± 0.3	−0.6 ± 0.3	+0.1 ± 0.2
12 months	−1.0 ± 0.6	+1.0 ± 0.3	−1.0 ± 0.6	−0.4 ± 1.3
Mean	−0.9 ± 0.6	+1.0 ± 0.7	−0.8 ± 0.5	−0.4 ± 0.9

Sign indicates the direction of the change (+, higher values; −, lower values) and an overall mean difference to control samples is provided for all tested preservation methods

Impacts of the tested preservation methods on isotope ratios of clams were surprisingly similar, particularly with respect to $\delta^{15}N$ where all preservation methods resulted in equally elevated values. Ethanol had the strongest impact on $\delta^{13}C$ values, which in part could relate to the lipid solving properties of ethanol (Syväranta et al., 2008b) as also the C:N ratios of those samples were affected. Similarly strong impact of freezing on the isotope values is more difficult to explain. Most likely this relates to mechanical effects and breakdown of cells allowing leaching of carbon

and nitrogen when thawed (Feuchtmayr & Grey, 2003; Dannheim et al., 2007). The duration of the preservation did not affect the δ^{15}N values, which remained constant throughout the experiment, and only formalin preserved δ^{13}C values became slightly (but significantly) lower with prolonged preservation time. Preservation did not increase the variation around the mean isotope values either in any case and in general the variation among individual clams was low. Our samples were collected at the same time from a small area and were of equal size so very little among-individual variation was expected. We are therefore confident that all the impacts seen on clam isotope values result from the different preservation methods.

Only two previous preservation studies testing impacts on clam tissues were identified in the literature (Sarakinos et al., 2002; Carabel et al., 2009). The results from those studies agree with our results as Sarakinos et al. (2002) reported 2.18 and 0.67‰ increase in δ^{13}C values after ethanol and formalin preservation, respectively, compared to our 2.2 and 1.3‰ increase. Similarly Carabel et al. (2009) reported significant increased in δ^{15}N values after ethanol–formalin preservation with an increase also around 1‰. However, Sarakinos et al. (2002) found opposing impacts on δ^{15}N values after ethanol and formalin preservation, and Carabel et al. (2009) found no or only minor impacts on δ^{13}C values. Both these studies used frozen samples as control treatment and therefore the results may not directly be comparable to ours. If frozen samples would have been used as control in this study, no impacts on δ^{15}N values and much lower impacts on δ^{13}C values (both higher and lower values) would have been noticed after preservation in ethanol, formalin or formalin–ethanol. In fact, such result would be in accordance with many previous studies using frozen samples as control.

Since all preservation methods had relatively low and, even more importantly, consistent impact on the δ^{15}N values ($\sim$1‰) and as nitrogen isotope values typically fractionate around 3‰ per trophic transfer (Peterson & Fry, 1987; Post, 2002), the δ^{15}N values of preserved clams can offer a suitable and rather reliable baseline indicator for retrospective studies in aquatic ecosystems. Our detailed results (Table 1) provide reliable means to account for preservation effects on sample δ^{15}N values allowing archived clams to be used for example to estimate historical trophic positions of consumers. The impacts on δ^{13}C values were greater, and carbon isotope values also fractionate less (<1‰) in food webs, making preserved clam samples less desirable for constructing historical food webs based on the δ^{13}C values. However, even though the effects of preservatives were greater on δ^{13}C values, the impacts were still rather consistent and provided that these observed impacts on carbon isotope values are accounted for, clams may offer an attractive baseline indicator organism for retrospective studies in aquatic ecosystems.

In conclusion, our results clearly illustrate that common preservation methods, including freezing, significantly affect the stable isotope values in sample materials (here clams) but our well controlled study also illustrates that these impacts are consistent and can be accounted for. Therefore, we conclude that preserved clams can indeed be used for stable isotope analyses but care has to be taken and the values need to be appropriately adjusted for preservation effects, as shown in this study. However, for studies not restricted to using preserved samples we advise to use only freshly dried samples and to avoid all unnecessary freezing and preserving for SIA. We also recognise a clear need for further taxa-specific, well controlled experiments of preservation impacts, including freezing, on stable isotope sample materials.

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References

Barrow, L. M., K. A. Bjorndal & K. J. Reich, 2008. Effects of preservation method on stable carbon and nitrogen isotope values. Physiological and Biochemical Zoology 81: 688–693.

Bosley, K. L. & S. C. Wainright, 1999. Effects of preservatives and acidification on the stable isotope ratios (^{15}N:^{14}N, ^{13}C:^{12}C) of two species of marine animals. Canadian Journal of Fisheries and Aquatic Sciences 56: 2181–2185.

Carabel, S., P. Verísimo & J. Freire, 2009. Effects of preservatives on stable isotope analyses of four marine species. Estuarine, Coastal and Shelf Science 82: 348–350.

Dannheim, J., U. Struck & T. Brey, 2007. Does sample bulk freezing affect stable isotope ratios of infaunal macrozoobenthos? Journal of Experimental Marine Biology and Ecology 351: 37–41.

Edwards, M. S., T. F. Turner & Z. D. Sharp, 2002. Short- and long-term effects of fixation and preservation on stable isotope values ($\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$) of fluid-preserved museum specimens. Copeia 4: 1106–1112.

Feuchtmayr, H. & J. Grey, 2003. Effect of preparation and preservation procedures on carbon and nitrogen stable isotope determinations from zooplankton. Rapid Communications in Mass Spectrometry 17: 2605–2610.

Fry, B., 2006. Stable Isotope Ecology. Springer, New York.

Kaehler, S. & E. A. Pakhomov, 2001. Effects of storage and preservation on the $\delta^{13}C$ and $\delta^{15}N$ signatures of selected marine organisms. Marine Ecology Progress Series 219: 299–304.

Kelly, B., J. B. Dempson & M. Power, 2006. The effects of preservation on fish tissue stable isotope signatures. Journal of Fish Biology 69: 1595–1611.

Mullin, M. M., G. H. Rau & R. W. Eppley, 1984. Stable nitrogen isotopes in zooplankton: some geographic and temporal variations in the North Pacific. Limnology and Oceanography 29: 1267–1273.

Ogawa, N. O., T. Koitabashi, H. Oda, T. Nakamura, N. Ohkouchi & E. Wada, 2001. Fluctuations of nitrogen isotopes of gobiid fish (*Isaza*) specimens and sediments in Lake Biwa, Japan, during the 20th century. Limnology and Oceanography 46: 1228–1236.

Peterson, B. J. & B. Fry, 1987. Stable isotopes in ecosystem studies. Annual Reviews in Ecology and Systematics 18: 293–320.

Post, D. M., 2002. Using stable isotopes to estimate trophic position: models, methods and assumptions. Ecology 83: 703–718.

Sarakinos, H. C., M. L. Johnson & M. J. Vander Zanden, 2002. A synthesis of tissue-preservation effects on carbon and nitrogen stable isotope signatures. Canadian Journal of Zoology 80: 381–387.

Sweeting, C. J., N. V. C. Polunin & S. Jennings, 2004. Tissue and fixative dependent shifts of $\delta^{13}C$ and $\delta^{15}N$ in preserved ecological material. Rapid Communications in Mass Spectrometry 18: 2587–2592.

Syväranta, J., M. Tiirola & R. I. Jones, 2008a. Seasonality in lake pelagic $\delta^{15}N$ values: patterns, possible explanations, and implications for food web baselines. Fundamental and Applied Limnology 172: 255–262.

Syväranta, J., S. Vesala, M. Rask, J. Ruuhijärvi & R. I. Jones, 2008b. Evaluating the utility of stable isotope analyses of archived freshwater sample materials. Hydrobiologia 600: 121–130.

Ventura, M. & E. Jeppesen, 2009. Effects of fixation on freshwater invertebrate carbon and nitrogen isotope composition and its arithmetic correction. Hydrobiologia 632: 297–308.